

Epithelial Phosphatidylinositol-3-Kinase Signaling Is Required for β -Catenin Activation and Host Defense against *Citrobacter rodentium* Infection[∇]

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Citrobacter rodentium infection of mice induces cell-mediated immune responses associated with crypt hyperplasia and epithelial β -catenin signaling. Recent data suggest that phosphatidylinositol-3-kinase (PI3K)/Akt signaling cooperates with Wnt to activate β -catenin in intestinal stem and progenitor cells through phosphorylation at Ser552 (P- β -catenin⁵⁵²). Our aim was to determine whether epithelial PI3K/Akt activation is required for β -catenin signaling and host defense against *C. rodentium*. C57BL/6 mice were infected with *C. rodentium* and treated with dimethyl sulfoxide (DMSO) (vehicle control) or with the PI3K inhibitor LY294002 or wortmannin. The effects of infection on PI3K activation and β -catenin signaling were analyzed by immunohistochemistry. The effects of PI3K inhibition on host defense were analyzed by the quantification of splenic and colon bacterial clearance, and adaptive immune responses were measured by real-time PCR (RT-PCR) and enzyme-linked immunosorbent assay (ELISA). Increased numbers of P- β -catenin⁵⁵²-stained epithelial cells were found throughout expanded crypts in *C. rodentium* colitis. We show that the inhibition of PI3K signaling attenuates epithelial Akt activation, the Ser552 phosphorylation and activation of β -catenin, and epithelial cell proliferative responses during *C. rodentium* infection. PI3K inhibition impairs bacterial clearance despite having no impact on mucosal cytokine (gamma interferon [IFN- γ], tumor necrosis factor [TNF], interleukin-17 [IL-17], and IL-1 β) or chemokine (CXCL1, CXCL5, CXCL9, and CXCL10) induction. The results suggest that the host defense against *C. rodentium* requires epithelial PI3K activation to induce Akt-mediated β -catenin signaling and the clearance of *C. rodentium* independent of adaptive immune responses.

Phosphatidylinositol-3-kinase (PI3K)/Akt signaling induces cell proliferative, growth, and survival signals in intestinal epithelial cells (IECs) (2, 17, 61). PI3K phosphorylates PIP2 to generate PIP3, which functions as a ligand to recruit pleckstrin homology (PH) domain-containing proteins to the inner surface of the cellular membrane. Chief among these is Akt, a serine/threonine kinase that is itself phosphorylated upon activation. Akt has emerged as a critical signaling node in mammalian cells and mediates the majority of PI3K's downstream effects. Among its many pro-proliferative functions, Akt signaling enhances mRNA stability and protein synthesis (via mTOR and S6 kinase) and the uptake of energy substrates (36). Activated Akt opposes apoptotic signaling by phosphorylating and sequestering bcl-xL/bcl-2-associated death promoter homolog (BAD) and Forkhead box (FOXO) factors in the cytoplasm. Recent findings suggest that activated Akt also acts in the nucleus by phosphorylating substrates, such as β -catenin, that increase the transcription of target genes associated with proliferation (e.g., cyclin D1 and c-Myc), tissue

remodeling (e.g., MMP7), and host defense (e.g., cryptdins, Muc2, and intestinal trefoil factor) (15, 18, 33).

Wnt/ β -catenin signaling is critical to the maintenance of intestinal crypt cell proliferation. Wnt ligands bind intestinal stem cells (ISCs) and crypt epithelial progenitor cells (PCs) and prevent glycogen synthase kinase 3 β (GSK3 β)-dependent N-terminal phosphorylation and proteosomal degradation of β -catenin (22, 29, 55, 60). When degradation is impaired by Wnt signaling, β -catenin translocates to the nucleus (a hallmark of activated canonical Wnt/ β -catenin signaling [43]), where it initiates the transcription of Wnt target genes such as the c-Myc and cyclin D1 genes (23, 29, 47). Upon division, ISCs and PCs give rise to rapidly cycling transit-amplifying cells (3, 5, 37). Previous studies showed that the deletion of the phosphatase and tensin homolog (PTEN), a negative regulator of PI3K, increases PI3K/Akt activation and enhances nuclear β -catenin accumulation (42). More recently, He et al. reported that PTEN deficiency increases PI3K/Akt activation, resulting in excessive proliferation and crypt fissioning within small bowel (SB) polyps (24). To investigate the interface between activated PI3K/Akt and β -catenin stabilization, researchers developed a phospho-specific antibody (Ab) that identifies β -catenin phosphorylated by Akt at Ser552 (P- β -catenin⁵⁵²) (24). Compared to N-terminal phosphorylation, which targets β -catenin for degradation, C-terminal phosphorylation at Ser552 is detected on nuclear β -catenin associated with the

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enhanced transcription of Wnt/ β -catenin target genes (18, 24). The staining of tissues from SB polyps showed P- β -catenin⁵⁵² within nuclei of dividing (activated) IECs and PCs in crypt bases and at points of crypt branching (24). Taken together, these results suggest that PI3K/Akt activation cooperates with Wnt to promote β -catenin signaling in IECs and PCs.

Overactive PI3K signaling occurs in a variety of tumor types, including breast, lung, ovarian, pancreatic, prostate, and colon cancers (8, 14, 20, 41, 46, 57, 62). Furthermore, defects in PTEN occur in a wide spectrum of human cancers (10). Recent studies by Dahan and colleagues suggested that PI3K/Akt signaling is upregulated in crypt IECs in chronic ulcerative colitis and active Crohn's disease (11). We recently reported that 5-aminosalicylic acid treatment impairs Akt-mediated β -catenin signaling and prevents the development of colitis-associated dysplasia (7). These data suggest that the activation of PI3K is an important step in the activation of IEC β -catenin signaling in dysplasia and cancer. As activating mutations of the p110 subunit of PI3K are among the most common mutations detected in all human cancers (27, 45, 46), several newly developed cancer therapeutics target this pathway (30, 35). Therefore, molecules that interfere with PI3K signaling may be chemopreventive in inflammation-induced cancer by inhibiting β -catenin signaling and impairing ISC and PC activation.

Despite literature examining PI3K and β -catenin in crypt maintenance, cell survival, and malignancy, little is known about its role in intestinal inflammation. This is especially important in infectious enterocolitis, where the maintenance of the epithelial barrier is critical to survival. *Citrobacter rodentium* is classified as an attaching and effacing (A/E) pathogen whose colonization of colon epithelial cells results in epithelial hyperplasia and mucosal inflammation (6). *C. rodentium* induces crypt hyperplasia, promotes PI3K recruitment to lipid rafts (51), and increases levels of nuclear β -catenin in crypt IECs (49, 50). Studies here address the possibility that efficient host defense responses to *C. rodentium* infection involve PI3K-induced β -catenin activation. Our results indicate that *C. rodentium* infection increases IEC levels of activated P-Akt and nuclear P- β -catenin⁵⁵². To determine the requirement for the PI3K regulation of epithelial β -catenin signaling in host defense, we inhibited PI3K signaling in mice inoculated with *C. rodentium*. The loss of PI3K signaling in infected mice reduced IEC Akt and β -catenin activation and attenuated IEC bromodeoxyuridine (BrdU) incorporation. The inhibition of PI3K signaling delayed bacterial clearance while not affecting inflammatory responses. Taken together, these results suggest that PI3K-mediated Akt and β -catenin signaling play critical roles in epithelial cell-specific host responses to enteric infection.

MATERIALS AND METHODS

Animals. Six- to eight-week-old C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were maintained within infection-controlled containment housing in the Northwestern University animal care facility. All experiments were approved by the Animal Care and Usage Committee of Northwestern University.

Induction of colitis and study design. *C. rodentium* strain DBS120 (48), a kanamycin-resistant strain (a gift from David Schauer, Boston, MA), was prepared by shaking at 37°C overnight in LB broth. The bacterial concentration was determined by measuring the absorbance at an optical density at 600 nm. CFU were confirmed through serial dilution and plating. Mice were fasted for 8 h prior

to inoculation with 10⁹ CFU in 200 μ l sterile phosphate-buffered saline (PBS). During the study, mice were allowed free access to food and drinking water. Mice were divided into 2 groups: group 1 received 200- μ l intraperitoneal (i.p.) injections of dimethyl sulfoxide (DMSO) diluted 1:4 in PBS (vehicle control) on days 5, 8, and 13 after infection, and group 2 received 1 mg (approximately 50 mg/kg of body weight) LY294002 (Sigma, St. Louis, MO) dissolved in DMSO diluted 1:4 in PBS. In separate studies, a parallel set of mice in group 2 received 0.1 mg/kg wortmannin (Sigma, St. Louis, MO) dissolved in DMSO diluted 1:5 in PBS on days 4, 6, 8, and 10. In studies to specifically examine interleukin-22 (IL-22) production, injections of DMSO or LY294002 were given on days 1, 3, and 5 after infection, and mice were sacrificed for analysis on days 4 and 7.

Histological and IHC analysis. A distal 1-cm piece of colon was removed from each mouse, fixed in 10% neutral buffered formalin, routinely processed, sectioned at 5 μ m, and stained with hematoxylin and eosin (H&E) for microscopic examination. Crypt lengths were measured by the micrometric sampling of at least 15 crypts per mouse. Histologic colitis scores were determined by measuring the severity of epithelial ulceration (0, none; 1, epithelial desquamation; 2, erosions with gaps of 1 to 10 IECs; 3, ulcers >10 IECs long), the extent of inflammatory cell infiltration (0, normal; 1, mild infiltration of the mucosa; 2, moderate to severe infiltration of the mucosa; 3, mild infiltration of the submucosa; 4, moderate to severe infiltration of the submucosa; 5, transmural infiltration into the serosa), and the extent of submucosal edema (0, none; 1, <200 μ m; 2, 200 to 450 μ m; 3, >450 μ m). BrdU, P-Akt, and P- β -catenin⁵⁵² immunohistochemical (IHC) staining was performed as previously described (32).

Immunofluorescence analysis. Paraffin-embedded tissue sections were subjected to antigen retrieval as described above for P- β -catenin⁵⁵². Slides were incubated with rabbit anti-Myc Ab (Millipore/Upstate, Temecula, CA) diluted 1:700 in PBS for 48 h at 4°C. After washes with PBS, horseradish peroxidase (HRP)-conjugated polymeric secondary antibody (EnVision anti-Rabbit HRP; Dako) was applied for 60 min at room temperature. Slides were fluorescently labeled with Alexa 488 via tyramide signal amplification according to the manufacturer's instructions (Invitrogen), washed with PBS, stained with Hoechst 33342 dye (Invitrogen), washed in distilled water, and mounted in aqueous medium. Slides were imaged with a Nikon C1 confocal microscope.

CFU counts. At selected time points, colons were removed from anus to cecum, and tissue and stool were homogenized by using a Polytron tissue homogenizer (Kinematica, Switzerland). Spleens were homogenized through a 100- μ m mesh filter. Homogenates were serially diluted and plated in duplicate onto MacConkey agar (Fluka, Steinheim, Germany) containing 40 μ g/ml kanamycin (Fluka) (*C. rodentium* strain DBS120 carries a kanamycin-resistant plasmid), and colonies were counted after 18 h of incubation at 37°C.

Real-time PCR analysis. Tissue RNA was isolated by using Trizol reagent (Invitrogen). Real-time PCR (RT-PCR) analysis was performed by using a qScript RT kit (Quanta, Gaithersburg, MD), a QuantiTect SYBR green PCR kit (Qiagen), and primers with an ABI 7500 real-time PCR system. Primers were designed to span genomic DNA intron junctions for the specific amplification of mRNA. Samples were normalized by using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Fold increases were calculated by using the $\Delta\Delta$ CT method. Primer sequences are available upon request.

Colon explant culture ELISA. One-centimeter pieces of distal colonic tissue were weighed, irrigated with PBS, shaken at room temperature in RPMI medium containing 50 μ g/ml gentamicin for 30 min, and distributed (0.05 g per well) into 24-well plates in 1 ml of RPMI medium supplemented with 5% fetal bovine serum, 50 μ g/ml gentamicin, and 1% penicillin-streptomycin-amphotericin B (Gibco, Grand Island, NY) for 20 h at 37°C. Supernatants were collected and stored at -80°C until protein quantification via enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN).

Statistical analysis. Data were analyzed with GraphPad Prism software by means of analysis of variance (ANOVA) followed by Tukey's range test when more than two groups were compared; otherwise, the Student *t* test was used. *P* values of ≤ 0.05 were considered statistically significant. Unless specified, values represent means \pm standard errors of the means (SEM). Unless specified, data are representative of at least 2 independent experiments.

RESULTS

***C. rodentium* colitis induces PI3K and β -catenin signaling in hyperplastic crypts.** *C. rodentium* induces pronounced colonic crypt hyperplasia in mice. We postulated that *C. rodentium* elicits epithelial proliferation by activating PI3K and β -catenin signaling. To examine this possibility, tissues were stained for

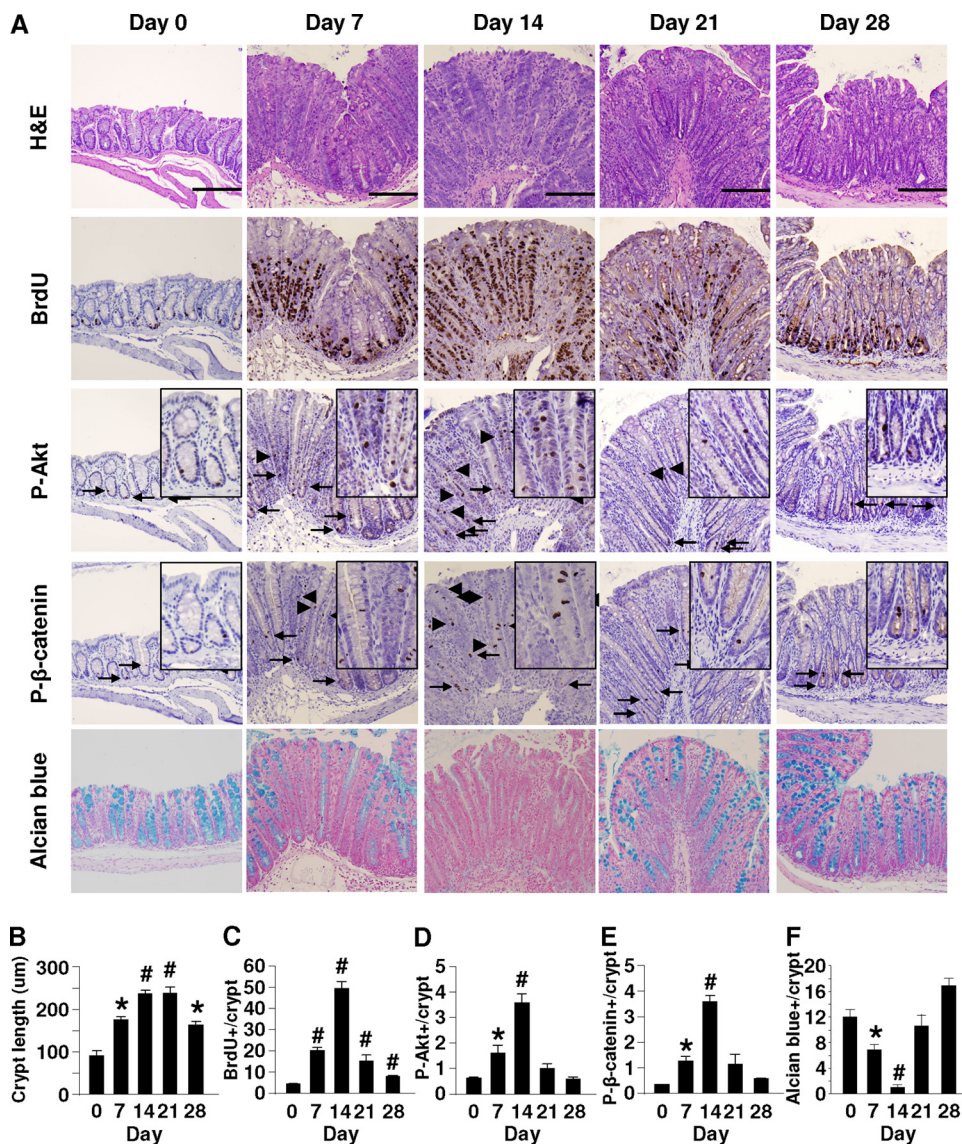


FIG. 1. *C. rodentium* induces epithelial hyperplasia and PI3K signaling. Colon tissue samples from controls (day 0) or mice inoculated with *C. rodentium* are shown. (A) From left to right are representative photomicrographs of tissues from infected mice at the specified time points stained with H&E and for BrdU, P-Akt, P-β-catenin⁵⁵², and alcian blue. Arrows indicate positively stained cells within crypt bases, and arrowheads indicate staining in the mid-crypt to upper crypt regions. (B to E) Infection resulted in crypt lengthening (B) as proliferation (BrdU incorporation) increased 6-fold (C) as well as increased PI3K signaling determined by the enumeration of P-Akt-positive epithelial cells/crypt (D) and P-β-catenin⁵⁵²-positive IECs/crypt (E). (F) Enumeration of alcian blue-positive cells/crypt demonstrates the progressive loss of goblet cells coinciding with increased PI3K signaling to day 14. Goblet cells repopulate crypts as the PI3K signal returns to baseline. Values are means ± SEM. Magnification, ×200 with ×400 inserts. Scale bar, 100 μm. *, $P \leq 0.02$; #, $P \leq 0.01$ (versus day 0).

BrdU incorporation, P-Akt, and P-β-catenin⁵⁵² (Fig. 1A). The results indicate that crypt lengths nearly doubled within 1 week of infection (baseline to day 7, 92 ± 10 to 176 ± 7 μm [mean ± SEM]) (Fig. 1B) and then peaked within weeks 2 and 3 (239 ± 14 μm). Coincident with crypt lengthening, numbers of cells staining for BrdU, P-Akt, and P-β-catenin⁵⁵² increased 3- to 5-fold within the first week, peaked by day 14, and declined thereafter (Fig. 1C to E). An examination of timed sections in the first 2 weeks suggested that *C. rodentium* induced IEC proliferation within the mid-crypt and upper crypt regions. In comparison, BrdU-stained cells receded from upper crypts 1 week later (day 21). Likewise, P-Akt and P-β-catenin⁵⁵² stain-

ing reached apical crypt regions by day 14 before diminishing. An examination of alcian blue staining indicated that numbers of goblet cells decreased during the first 2 weeks of infection (Fig. 1F), as previously described (4, 34). To test whether the increase in BrdU and PI3K/β-catenin signaling was simply an effect of the increased crypt length, numbers of positive cells were calculated based on average crypt lengths. Increased crypt lengths in inflamed mice reduced the absolute differences in counts, but data remained consistent with total crypt counts (data not shown). These results raised the possibility that IEC proliferative responses to *C. rodentium* were induced by PI3K and β-catenin signaling in the mid-crypts and upper crypts.

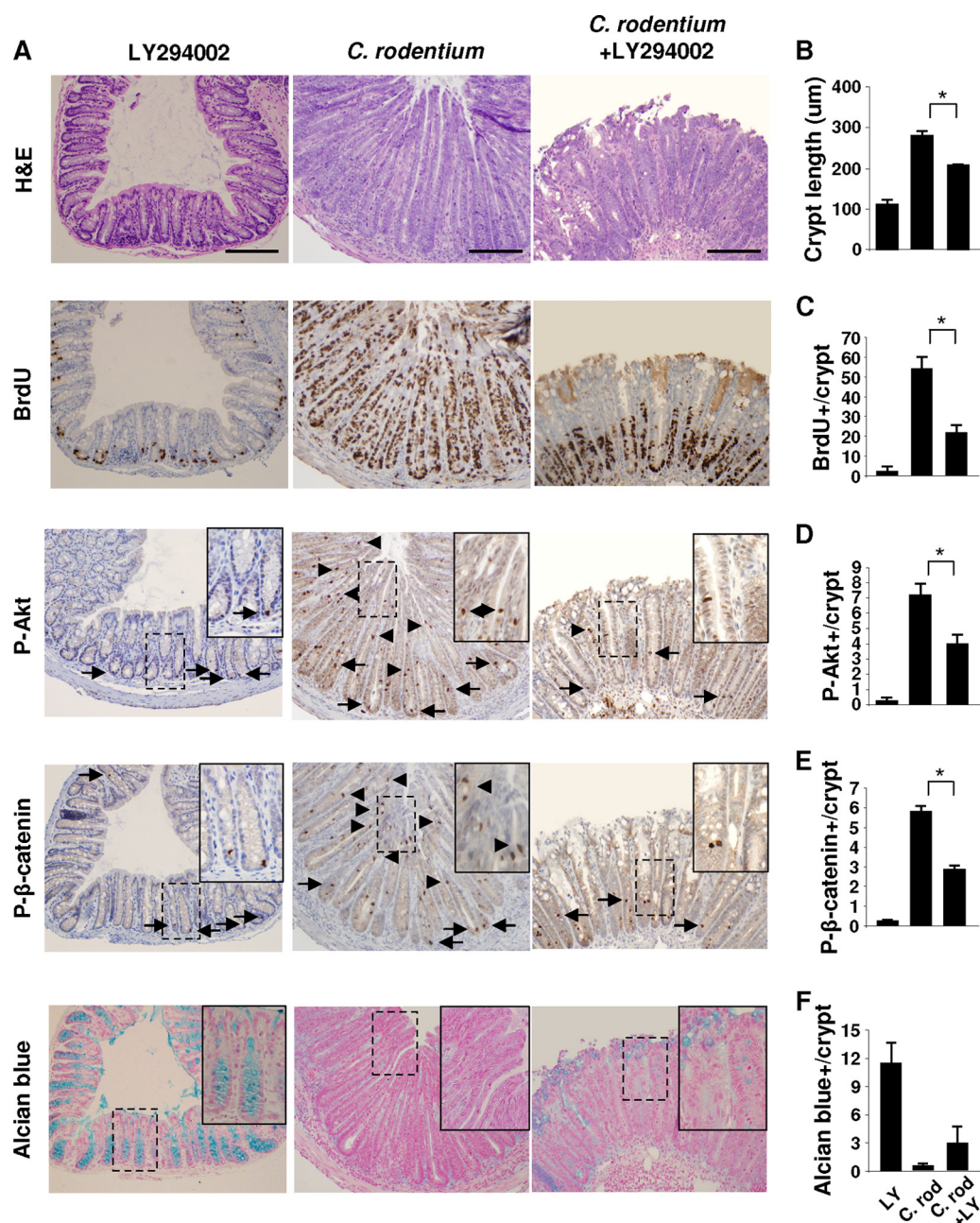


FIG. 2. PI3K inhibition impairs *C. rodentium*-induced Akt and β -catenin signaling and attenuates epithelial cell proliferation. The distal colon was removed from C57BL/6 mice 14 days after oral infection with *C. rodentium* (C. rod) treated with either DMSO (vehicle control) (middle) or LY294002 (LY) (right). Left panels represent uninfected mice treated with LY294002. (A) Colonic tissue stained with H&E and for BrdU, P-Akt, P- β -catenin⁵⁵², and alcian blue. Arrows indicate positively stained cells within crypt bases, and arrowheads indicate staining in the mid-crypt to upper crypt regions. (B to F) LY294002 treatment alone had no effect relative to baseline but impaired *C. rodentium*-induced crypt lengthening, PI3K signaling, proliferation, and goblet cell depletion. Values are means \pm SEM. Magnification, $\times 200$ with $\times 400$ inserts. Scale bar, 100 μ m. *, $P < 0.01$.

PI3K signaling is required for P- β -catenin⁵⁵² expression and crypt proliferation in *C. rodentium*-induced colitis. To examine the role of PI3K signaling in epithelial responses to *C. rodentium* infection, infected mice were treated with vehicle (DMSO) or the selective PI3K inhibitor LY294002 during the first 2 weeks of infection (Fig. 2A). A third group of uninfected mice was treated with an identical dosing schedule of LY294002 over 2 weeks to assess the effect of PI3K inhibition

on normal IEC turnover. Morphometric analysis data (Fig. 2B) indicate that crypt lengths were unaffected by the PI3K blockade in uninfected mice. In contrast, PI3K inhibition reduced crypt elongation by 36% in infected mice. IHC results show that PI3K inhibition had no effect on the distribution of staining in uninfected mice but attenuated the vertical expansion of BrdU, P-Akt, and P- β -catenin⁵⁵² staining into the upper crypts of *C. rodentium*-infected mice. Cell counting of BrdU-, P-Akt-,

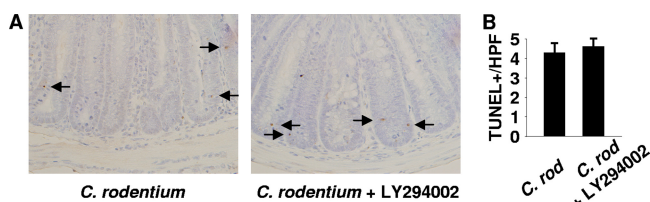


FIG. 3. PI3K inhibition does not affect epithelial cell apoptosis. (A) Distal colon sections from mice infected 14 days earlier with *C. rodentium* and treated with either DMSO or LY294002 were stained for apoptotic cells by the TUNEL technique. (B) Enumeration of TUNEL-positive epithelial cells per $\times 400$ field. Values are means \pm SEM. HPF, high-power field.

and P- β -catenin⁵⁵²-stained sections demonstrated that PI3K inhibition reduced numbers of BrdU-positive cells by 67% (Fig. 2C) and reduced active Akt and β -catenin signaling by 50% and 42%, respectively (Fig. 2D and E). Additionally, we observed that reductions in goblet cell numbers in *C. rodentium*-infected mice were partially reversed, as alcian blue-stained cells reappeared in the upper crypts of LY294002-treated mice (Fig. 2F). To confirm that changes in PI3K signaling measured per crypt were due solely to impaired proliferation (i.e., fewer numbers of IECs), BrdU-, P-Akt-, and P- β -catenin⁵⁵²-positive cells were enumerated on a per-IEC basis. The results were similar to crypt counts revealing that PI3K inhibition reduced the absolute number of proliferating epithelial cells with β -catenin signaling (data not shown). Finally, to test whether the PI3K inhibition affected levels of IEC apoptosis, sections were stained by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL). The data showed no difference in apoptotic indices after LY294002 treatment (Fig. 3). Taken together, these data suggest that PI3K activation induced IEC proliferation via the Akt-dependent phosphorylation of β -catenin in *C. rodentium* colitis, altering IEC death. The net result was to increase crypt lengths.

Functional activity of β -catenin is dependent on PI3K-mediated Ser552 phosphorylation. Our data for both human inflammatory bowel disease and experimental IL-10^{-/-} murine colitis (7, 32) as well as data reported previously by Fang et al. (18) suggest that Ser552 phosphorylation of β -catenin stimulates the transcriptional expression of Wnt/ β -catenin target genes. The c-Myc and cyclin D1 genes are Wnt/ β -catenin target genes involved in cell proliferation (23, 59). In recent studies, Muncan and colleagues reported that c-Myc protein expression denotes proliferative transit amplifying cells in the mid-crypt (39). To assess the functional consequences of PI3K inhibition for epithelial c-Myc and cyclin D1 expression, colon sections were stained for c-Myc and cyclin D1 (Fig. 4). In uninfected mice, c-Myc and cyclin D1 stainings localized to IECs immediately above (c-Myc) or within (cyclin D1) the crypt base. In response to infection, both c-Myc and cyclin D1 levels increased throughout the mid-crypts and upper crypts. The data also revealed that LY294002 treatment inhibited the expression of both proteins in the mid- and upper crypts. These findings suggest that *C. rodentium* infection induced the expression of c-Myc and cyclin D1 in transit, amplifying cells through a PI3K-dependent process. Together, the data suggest that *C. rodentium*-induced IEC PI3K activation stimulated ep-

ithelial cell proliferation by stimulating Akt and β -catenin signaling.

PI3K signaling is required for host defense against *C. rodentium*. To test the role of PI3K signaling in host responses to *C. rodentium*, body weights and *C. rodentium* bacterial loads in control and LY294002-treated mice were measured up to 28 days after infection. Infection caused transient weight loss at day 4 (average body weight decreased by 0.5%), followed by weight gain (Fig. 5). Infected mice treated with LY294002 failed to gain weight at day 7, followed by further weight loss that continued until day 16, whereupon a gradual recovery ensued. To determine whether toxicity from LY294002 itself caused the weight loss, uninfected wild-type (WT) mice were treated with 3 doses of LY294002, and weights were compared to those of control infected mice. LY294002-treated uninfected mice gained weight in parallel with untreated mice. We then considered the possibility that weight loss in LY294002-treated infected mice resulted from a prolonged bacterial infection. Consistent with this interpretation, we found that DMSO- and LY294002-treated mice had comparable colonic and splenic bacterial burdens at day 7, but LY294002 treatment delayed the clearance of colonic bacteria at both day 14 (4-fold more CFU) and day 18 (300-fold more CFU) (Fig. 6A), which correlated with the weight divergence (day 10) between the two groups. Histological findings for LY294002-treated mice suggested that attenuated PI3K signaling impaired epithelial restitution (Fig. 2), resulting in more severe, prolonged ulceration, and impaired barrier function (Fig. 7). We then tested whether compromised mucosa correlated with enhanced bacterial translocation. Analysis of splenic *C. rodentium* levels revealed that infected control mice cleared splenic bacteria by day 18. In comparison, splenic bacterial burdens in LY294002-treated mice remained elevated at days 14 and 18, with clearance at day 21 (Fig. 6B). Together, these data support the hypothesis that epithelial PI3K signaling helps to prevent *C. rodentium* bacterial invasion.

Host adaptive immune responses are not affected by treatment with LY294002. Previous studies indicated that cytokine-mediated (13, 21, 31, 52, 53, 56, 58) and chemokine-mediated (54) adaptive immune responses promote host defense against *C. rodentium*. To determine the effect of PI3K inhibition on the adaptive mucosal host defense against *C. rodentium*, mucosal Th1, Th17, and macrophage cytokine levels, as well as chemokine responses, in infected mice were assessed. Data in Fig. 8A show that gamma interferon (IFN- γ), tumor necrosis factor (TNF), IL-17, and CXCL9 mRNA levels peaked within 14 days of *C. rodentium* infection. Furthermore, treatment with LY294002 during this period did not affect the induction of these inflammatory mediators. In fact, cytokine and chemokine mRNA levels for all genes assessed increased and/or remained elevated in LY294002-treated mice, including CXCL1, CXCL5, CXCL10, IL-1 β , and inducible nitric oxide synthase (iNOS) (data not shown). Protein quantification by colon explant culture ELISA (Fig. 8B) at day 14 revealed that *C. rodentium* infection also increased cytokine and chemokine protein levels independent of PI3K. Interestingly, mucosal IFN- γ , TNF, CXCL9, CXCL10, and iNOS mRNA levels increased after LY294002 treatment was stopped. An examination of tissues during this period revealed that tissues remained inflamed (histologic colitis score of 5.8 ± 0.2 versus 6.3 ± 0.3

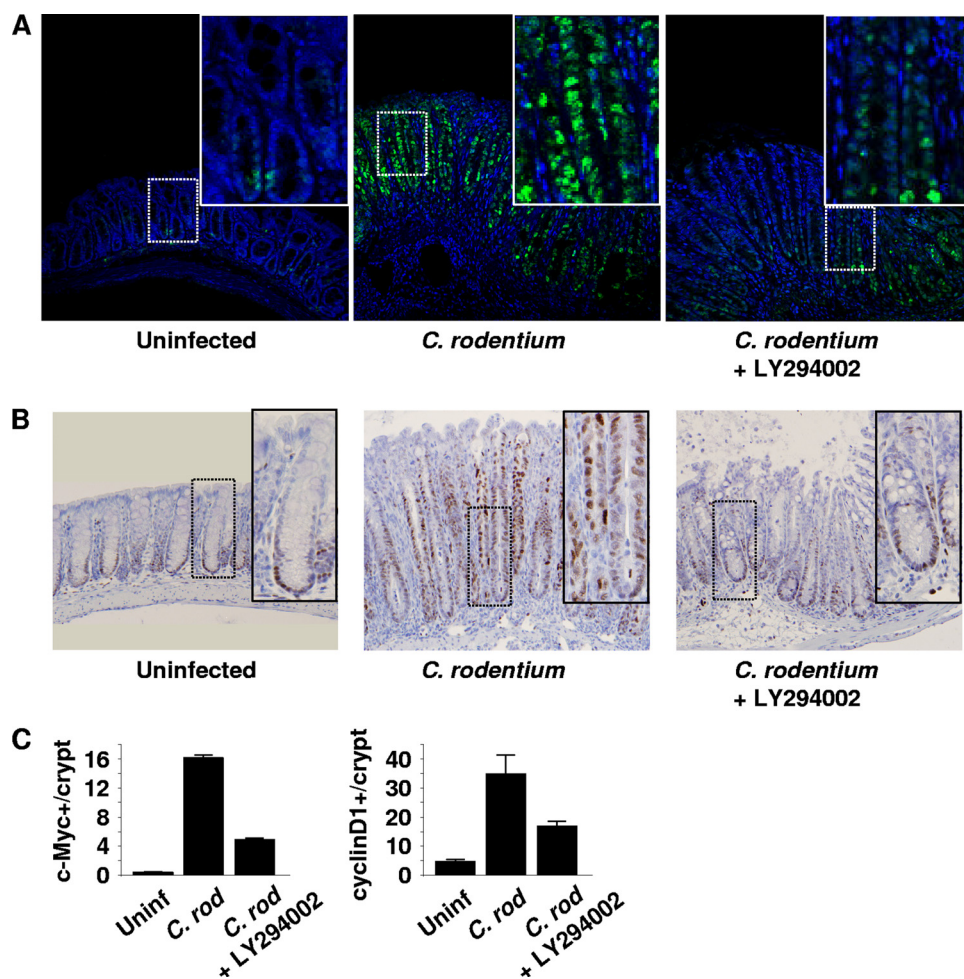


FIG. 4. Inhibition of PI3K signaling reduces β -catenin signaling in *C. rodentium* colitis. (A and B) Colon sections from uninfected (day 0) and *C. rodentium*-infected mice treated with DMSO or LY294002 (day 14) were processed and stained to assess c-Myc (green) expression (A) and cyclin D1 (brown) expression (B). (C) Enumeration of c-Myc- and cyclin D1-positive epithelial cells per crypt. Values are means \pm SEM. Magnification, $\times 200$ with $\times 400$ inserts.

for *C. rodentium*-infected mice versus *C. rodentium*-infected mice with LY294002 treatment; the *P* value was not significant) (Fig. 7B). One possibility suggested by assessments of bacterial counts (Fig. 6) was that mucosal inflammation remained elevated to allow host defenses to recover from PI3K inhibition.

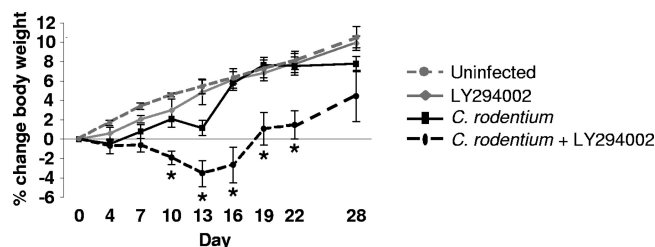


FIG. 5. Inhibition of PI3K signaling increases and prolongs weight loss. *C. rodentium* infection caused transient weight loss at day 4 with recovery by day 7 and subsequent weight gain. The blockade of PI3K signaling worsened weight loss that did not recover until day 19 and was significantly reduced until day 28. *, *P* < 0.01 versus *C. rodentium* (*n* = 4 to 8 mice for each group).

To test whether the increased cytokine and chemokine levels seen for LY294002-treated infected mice were due to LY294002, colon mRNA from uninfected mice that received 3 doses of LY294002 was compared to that from control uninfected mice. The data showed no increase in cytokine/chemokine mRNA levels after LY294002 treatment (data not shown).

Recently, IL-22 was implicated as a major determinant during the first week of mounting host defenses to *C. rodentium* (63). We considered this mechanism of host defense to be relevant because the IL-22 receptor is expressed on epithelial cells (1). Data in Fig. 9 show that at both days 4 and 7 after infection, IL-22 induction was unaffected.

Finally, we considered that PI3K inhibition may be affecting innate immune signaling, including antimicrobial functions of paneth cells and enterocytes. Data in Fig. 8C show that both Reg3 γ (Paneth cells) and MMP-7 (enterocytes) inductions were unaffected. In fact, an early enhanced induction of Reg3 γ mRNA (day 7) and prolonged induction of MMP-7 were noted. These data support the notion that PI3K signaling is critical to epithelial host defenses independent of adaptive inflammatory responses.

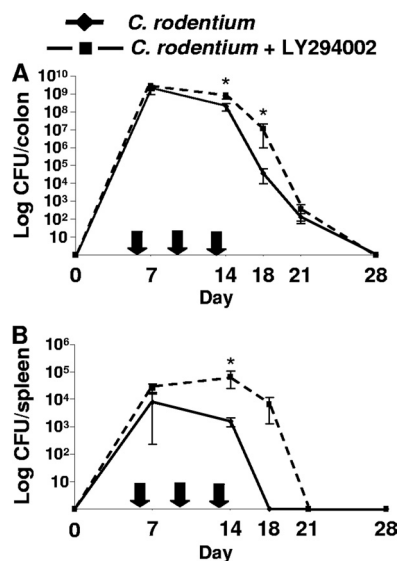


FIG. 6. PI3K signaling is required for clearance of *C. rodentium*. *In vivo* colonization and clearance dynamics were monitored from day 7 after inoculation with *C. rodentium* to day 28. (A) Mice receiving LY294002 showed prolonged colonization within the colon and shedding of bacteria in the stool, demonstrating that the PI3K inhibition reduces the ability to clear *C. rodentium* infection. (B) PI3K inhibition resulted in persistence of systemic infection within the spleen. Arrows indicate time points for LY294002 injection. *, $P < 0.05$ versus *C. rodentium* ($n = 3$ to 4 mice for each group, representative of 3 separate experiments).

Alternative PI3K inhibition with wortmannin corroborates impaired bacterial clearance with LY294002. Although we have demonstrated that PI3K inhibition by LY294002 treatment impairs mucosal host defenses by inhibiting epithelial cell proliferative responses while sparing host inflammatory responses, it remained possible that LY294002 was acting through a mechanism independent of the epithelial compartment or independent of PI3K. Therefore, we performed a similar set of experiments interrupting PI3K signaling with the irreversible PI3K inhibitor wortmannin. Morphometric analysis data (Fig. 10A) indicate that crypt lengths were unaffected by the PI3K blockade with wortmannin in uninfected mice. In contrast, PI3K inhibition reduced crypt elongation by 43% in infected mice, consistent with the impaired epithelial proliferative responses seen for LY294002-treated mice. Furthermore, wortmannin treatment resulted in a wasting state similar to that with LY294002 while having no effect on uninfected mice (Fig. 10B). As expected, colonic bacterial clearance was delayed at day 18, and systemic levels of bacteria were greater at day 14 and remained present at day 18 (Fig. 10C). To measure whether wortmannin spared host adaptive immune responses in a manner similar to that of PI3K inhibition with LY294002, colonic mRNA levels of IFN- γ , CXCL9, and iNOS were measured at days 14 and 18. Data in Fig. 10D show that PI3K inhibition did not affect the induction of these proinflammatory genes. These data corroborate findings for LY294002-treated mice and suggest that PI3K-mediated signaling plays a critical role in epithelial cell-specific host responses to enteric infection.

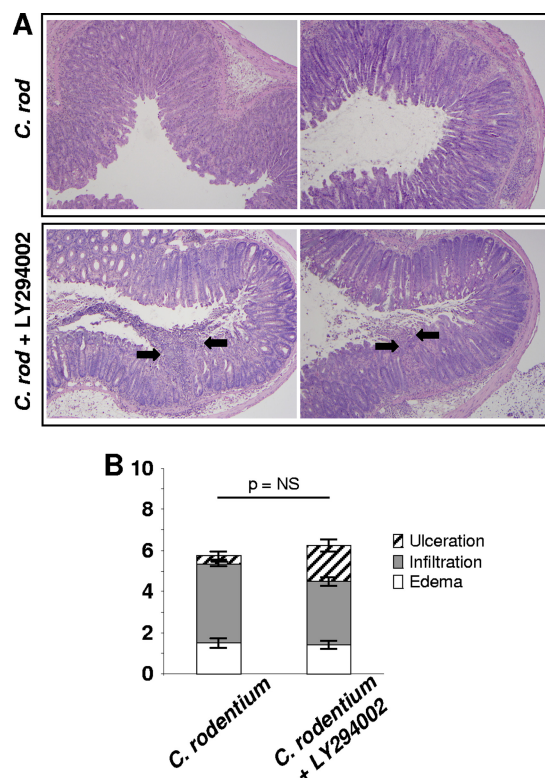


FIG. 7. Interruption of PI3K signaling disrupts the epithelial barrier. (A, top) Two separate representative distal colons stained with H&E demonstrating the expected crypt hyperplasia with relatively intact epithelial lining 14 days after infection with *C. rodentium*. (Bottom) Two separate representative distal colons in mice infected with *C. rodentium* and treated with LY294002. PI3K inhibition of host epithelial cell responses resulted in an increased disruption of the epithelial cell barrier and frank ulceration. Arrows indicate ulcer edges. Magnification, $\times 100$. (B) Histological scores for ulceration, inflammatory cell infiltration, and edema were assessed in colonic tissue samples from infected mice treated with DMSO or LY294002.

DISCUSSION

The present study proposes a novel view of the role of PI3K signaling in mediating epithelial responses to *C. rodentium*. Previous studies suggested that PI3K signaling induces epithelial proliferation in response to growth factor, TNF, and Toll-like receptor 4 ligation (16, 19, 25, 36). Data from epithelial cell coculture studies indicate that mucosal T cells from Crohn's disease tissue specimens stimulate IEC PI3K signaling (11, 12). The results presented here show that IEC PI3K signaling was induced during the period of *C. rodentium*-induced inflammation associated with crypt elongation and enhanced IEC proliferation (Fig. 1 and 2). Reductions in crypt elongation and IEC proliferation in LY294002-treated mice indicated that PI3K signaling was required for the proliferative responses induced by *C. rodentium*. Proliferative responses may be pathogen driven to facilitate an increased surface area for *C. rodentium* colonization. However, we found that blocking PI3K signaling/proliferation increased the pathogen burden and prolonged mucosal inflammation (Fig. 6 to 8). Thus, our findings are consistent with the notion that *C. rodentium*-stimulated IEC PI3K signaling induces proliferation as a compo-

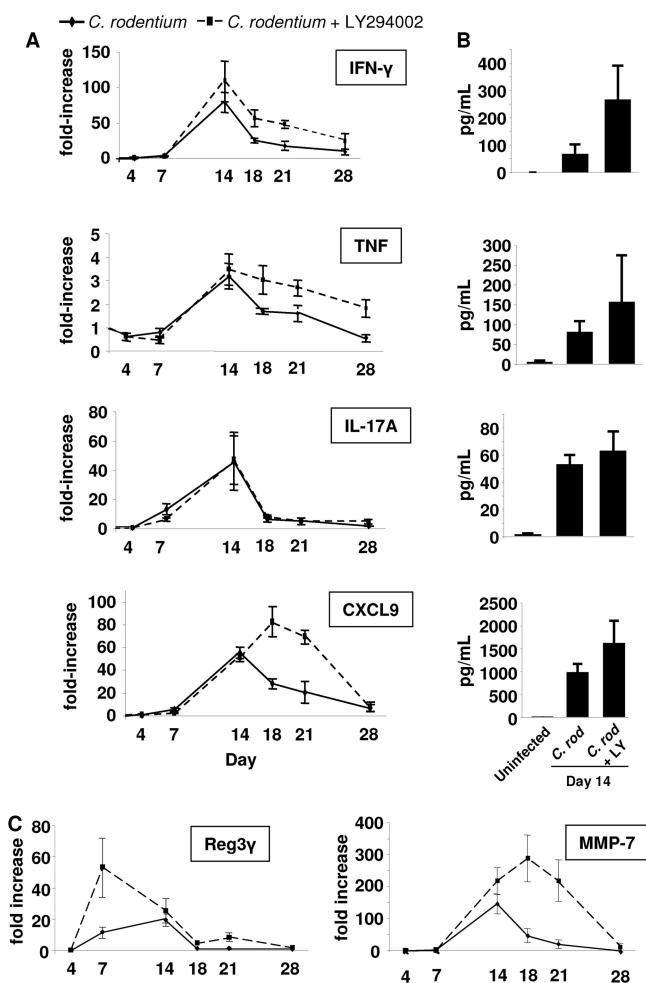


FIG. 8. PI3K is dispensable for adaptive and innate immune responses to *C. rodentium*. (A) Time course of mRNA (adaptive immunity) isolated from the distal colon after *C. rodentium* infection alone or with LY294002 treatment. (B) Distal colon segments were removed 14 days after infection, with and without LY294002 treatment; washed; and cultured overnight for direct cytokine analysis by ELISA corresponding to Fig. 8A mRNA. (C) Time course of mRNA (innate immunity) isolated from the distal colon after *C. rodentium* infection alone or with LY294002 treatment ($n = 3$ to 4 mice per group).

nent of mucosal host defenses needed to eliminate infection and resolve mucosal inflammation.

Data from our group (7, 24) and others (18) suggest that PI3K signaling serves to enhance Wnt-mediated β -catenin activation. Studies with PTEN-deficient mice suggested that enhanced PI3K signaling in IECs led to SB polyp formation. The detection of IECs with nuclear P- β -catenin⁵⁵² at points of crypt fissioning suggested that the Akt-mediated phosphorylation of β -catenin enhanced nuclear stabilization and activation. The anti-P- β -catenin⁵⁵² Ab employed in these studies was used here to visualize cells with a C-terminal phosphorylation of nuclear β -catenin in crypts of mice with *C. rodentium* infection. Sellin et al. previously reported enhanced levels of P- β -catenin⁵⁵² in *C. rodentium* colitis (49, 50). Studies here suggested that the induction of P- β -catenin⁵⁵² in crypts was regulated by PI3K signaling. In addition, the data were consistent with the notion that PI3K-mediated β -catenin activation

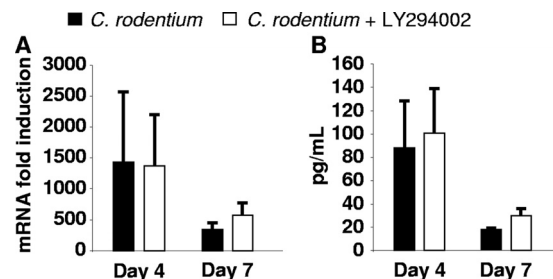


FIG. 9. PI3K inhibition does not reduce IL-22 production. PI3K inhibition does not affect either mRNA (A) or colon explant culture (B) ELISA measurements of IL-22 production 4 and 7 days after infection with *C. rodentium*.

played a key role in mounting robust crypt epithelial proliferative responses during *C. rodentium* infection. The localization of cells with nuclear P- β -catenin⁵⁵² in the upper crypts of infected mice suggests that PI3K signaling induced β -catenin activation in PCs within transit-amplifying populations. We postulate that PI3K signaling in upper crypt IECs was induced by inflammatory signals received from the local environment. Of the many downstream functions of PI3K/Akt signaling (protein stabilization and reduced apoptosis, etc.), we suspect that Akt interacts in a multicomponent complex with β -catenin in the nucleus to enhance activation. In this model, we suspect that Akt activation did not promote the nuclear localization of β -catenin, which occurs during Wnt signaling. Rather, our results suggest that β -catenin phosphorylation by Akt in the nucleus initiates the transcription of pro-proliferative genes (e.g., the c-Myc and cyclin D1 genes). This signal cooperates with effects of Akt in the cytosol (e.g., mTOR activation) that promote cell cycle progression and replication. Together, the data suggest that inflammatory signals derived from the surrounding milieu promote β -catenin signaling through a PI3K-dependent pathway.

The data presented show that PI3K inhibition was associated with increased systemic levels of *C. rodentium*. Because it is not possible to confirm unperturbed functions of all adaptive pro-inflammatory pathways (13, 21, 52–54, 56, 58) and innate host defense factors (e.g., cathelicidin and trefoil factor) (15, 26) or unknown effects of LY294002 and wortmannin, it remains possible that systemic PI3K inhibition may have contributed to the increased systemic bacterial load. However, the preservation of Th1, Th17, macrophage, paneth cell, epithelial cell, and chemokine mRNA levels suggested that PI3K inhibition did not impair mucosal inflammatory responses. Rather, we postulate that PI3K inhibition reduced epithelial proliferative responses and barrier function. This hypothesis is consistent with results reported previously by Cario and colleagues, who found that the inhibition of IEC PI3K/Akt signaling caused a disruption of tight junctions following Toll-like receptor 2 stimulation (9). Similarly, Resta-Lenert and Barrett found previously that PI3K inhibition reversed the protective effect of probiotics on barrier function in epithelial cell monolayers (44). The findings presented here add to the potential mechanisms for the role of PI3K signaling in epithelial barrier integrity. The data suggest that PI3K signaling may enhance mucosal barrier function in infectious colitis by promoting epithelial restitution.

A/E-type enteric pathogens such as enteropathogenic and

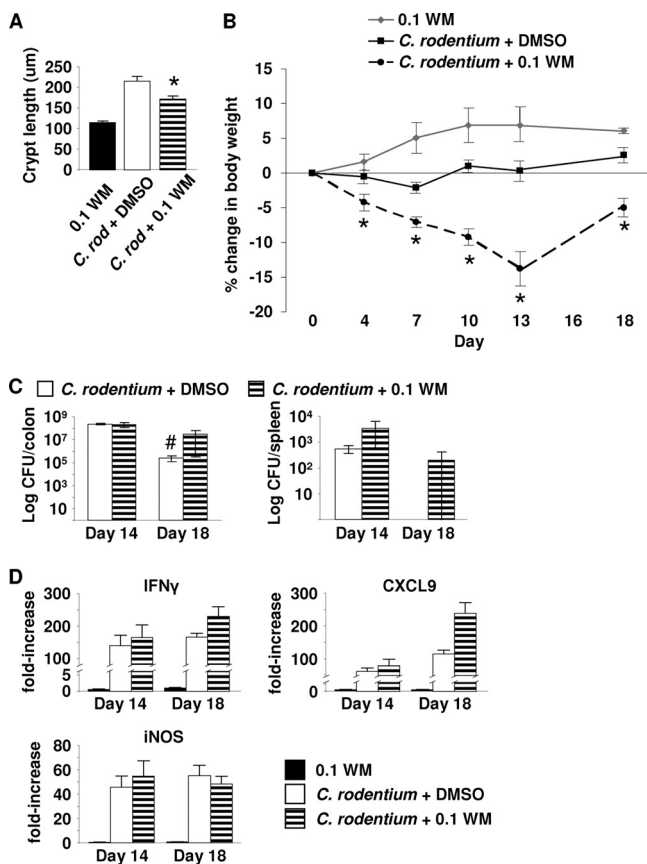


FIG. 10. PI3K inhibition with wortmannin (WM) impairs the host defense against *C. rodentium*. The distal colon was removed from C57BL/6 mice 14 or 18 days after oral infection with *C. rodentium* treated with either DMSO (vehicle control) or 0.1 mg/kg wortmannin (see Materials and Methods). (A) Wortmannin treatment alone had no effect relative to baseline but impaired *C. rodentium*-induced crypt lengthening. (B) *C. rodentium* infection caused transient weight loss with recovery by day 7 and subsequent weight gain. The blockade of PI3K signaling worsened weight loss that did not begin to recover until day 16. (C) Mice receiving wortmannin showed prolonged colonization within the colon and shedding of bacteria in the stool as well as a persistence of systemic infection within the spleen, demonstrating that PI3K inhibition reduces the ability to clear *C. rodentium* infection. (D) mRNA levels at days 14 and 18. Values are means \pm SEM. *, $P < 0.05$ versus *C. rodentium* plus DMSO; #, $P < 0.01$ versus day 14 ($n = 5$ to 8 mice per group).

enterohemorrhagic *Escherichia coli* strains generate substantial global morbidity (e.g., infantile diarrhea and hemolytic-uremic syndrome) and mortality, resulting in as many as 1 million deaths per year worldwide (28, 38, 40). Elucidating the mechanisms required for the host defense against A/E pathogens may impact global health. Data here utilize *C. rodentium*, an A/E murine pathogen, to show that the PI3K-dependent activation of β -catenin and subsequent epithelial proliferative responses are required for the normal host defense against *C. rodentium* colitis. In the current study, it remains unclear whether or not the eventual clearance of bacteria was due to a restitution of PI3K signaling after the cessation of LY294002 treatment or if enhanced inflammatory responses were enough to compensate for impaired epithelial signaling. Regardless, given the global morbidity and mortality associated with A/E

enteric pathogens and the fact that PI3K signaling may be targeted in future chemotherapeutic agents (35), it is crucial to better understand its role in nonmalignant inflammatory processes.

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